0022-3565/98/2843-0838$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 1998 by The American Society for Pharmacology and Experimental Therapeutics
JPET 284:838–846, 1998

Vol. 284, No. 3
Printed in U.S.A.

Coronary Vasorelaxation by Nitroglycerin: Involvement of Plasmalemmal Calcium-Activated K^+ Channels and Intracellular Ca^{++} Stores

SAJIDA A. KHAN, NICOLE R. HIGDON and KAUSHIK D. MEISHERI

Cardiovascular Pharmacology, Pharmacia & Upjohn, Inc., Kalamazoo, Michigan

Accepted for publication November 14, 1997 This paper is available online at http://www.jpet.org

ABSTRACT

This study investigated nitroglygerin (NTG) relaxations in isolated dog coronary artery in comparison with other vascular preparations. Under maximal PNU-46619 precontraction, the coronary artery was significantly more sensitive to NTG than mesenteric artery, mesenteric vein and saphenous vein. In the coronary artery, NTG (1–100 nM) produced relaxations with EC_{50} = 9.4 nM. In KCl-contracted arteries (20–80 mM KCl), relaxation by NTG was progressively reduced. Relaxation responses to NTG also were inhibited significantly by potent calcium-activated K^+ (BK) channel blockers, charybdotoxin (100 nM) and iberiotoxin (200 nM), but not by K_{ATP} blockers such as PNU-37883A (10 μM) or PNU-99963 (100 nM). Nitric oxide (0.1–30 nM) and acetylcholine (3–300 nM) also produced relaxations which were significantly attenuated by the BK blockers. In further experiments, NTG (1–100 nM) produced inhibition of PNU-46619-induced SR $[Ca^{++}]_i$ release, with an IC_{50} of 8.5 nM, which was not affected by charybdotoxin. Furthermore, P1075 (50 nM), a K_{ATP} opener, did not inhibit agonist-stimulated SR $[Ca^{++}]_i$ release. Ryanodine (10 μM), which acts on SR Ca^{++} release channels, did not alter NTG relaxations, whereas thapsigargin (0.1 μM), a selective inhibitor of SR Ca^{++}-ATPase pump, produced pronounced inhibition of NTG relaxations. These results suggest that NTG, in the therapeutic concentration range, produces coronary relaxation primarily via two cellular mechanisms: plasmalemmal BK channel activation and stimulation of SR Ca^{++}-ATPase to produce increased SR Ca^{++} accumulation. These two mechanisms apparently are equally important and act together to produce a unique vasorelaxation profile demonstrated by NTG-type coronary vasodilators.

Organic nitrates including NTG are established, potent vasodilators used for the control and treatment of angina, myocardial infarction and congestive heart failure. Vasorelaxation responses to nitro-vasodilators are mediated *via* the active intermediate NO which causes activation of soluble guanylate cyclase resulting in elevation of cyclic GMP levels (Ignarro and Kadowitz, 1985). During the years, a variety of agents that increase cyclic GMP levels (such as nitroglycerin, sodium nitroprusside, isosorbide dinitrate, nicorandil, atrial natriuretic factor, cyclic GMP phosphodiesterase inhibitors) have been used to probe different cellular calcium homeostasis mechanisms as targets for the actions of the cyclic GMP pathway (Lincoln, 1989).

Two generalizations can be made regarding the vascular actions of agents working *via* the cyclic GMP pathway. First, these agents produce preferential relaxation of agonist-induced contractions *versus* high K^+ depolarization-induced contractions (Karaki *et al.*, 1986; Taylor and Meisheri, 1986). Consistent with this, several studies have pointed out the role of membrane hyperpolarization, specifically the role of K^+ channel activation, in vasorelaxation by cyclic GMP-elevating agents (Tare *et al.*, 1990; Taniguchi *et al.*, 1993; Khan *et al.*, 1993). Second, these agents produce inhibition of agonist-stimulated release of intracellular Ca^{++} from sarcoplasmic reticulum stores (SR Ca^{++} release) (Hester, 1985; Meisheri *et al.*, 1986). In support of this, it has been demonstrated that the SR Ca^{++}-ATPase regulatory protein, phospholamban, is a good substrate for cyclic GMP-dependent protein kinase both *in vitro* and in intact smooth muscle cells (Lincoln and Cornwell, 1993). Additional mechanisms also have been reported, *e.g.*, inhibition of phospholipase C or activation of the plasmalemmal Ca^{++} extrusion pump, in the vascular smooth muscle actions of cyclic GMP (Hirata *et al.*, 1990; Yoshida *et al.*, 1991). Most data, however, support the contribution of the two key mechanisms above, *i.e.*, K^+ channel activation-mediated hyperpolarization which in turn lim-

Received for publication August 28, 1997.

ABBREVIATIONS: BK, calcium-activated K^+ channels or Maxi K channels; K_{ATP}, ATP-sensitive K^+ channel; CRC, concentration response curve; PSS, physiological salt solution; ACh, acetylcholine; NTG, nitroglycerin; RY, ryanodine; TG, thapsigargin; SR, sarcoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NO, nitric oxide; MeB, methylene blue; ChTX, charybdotoxin; IbTX, iberiotoxin; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

its the sarcolemmal Ca^{++} influx, and inhibition of agonist-stimulated SR Ca^{++} release.

Although the coronary artery is the primary target tissue for the antianginal actions of NTG, most mechanistic work is based on the use of vascular preparations other than the coronary artery. This, in turn, has resulted in the use of NTG concentrations (>100 nM) that are well above the known therapeutic NTG concentrations of approximately 10 nM (He *et al.*, 1996; Wei and Reid, 1979). In addition, the relative contributions of the two above-mentioned major mechanisms in the pharmacological actions of NTG remain unknown. So, the goals of this study were: 1) to investigate NTG relaxations in therapeutically relevant tissue, *i.e.*, coronary artery, with particular emphasis on studying NTG relaxations in the therapeutically relevant concentration range of 3 to 30 nM, 2) to investigate the relative contributions of the plasmalemmal K^+ channel mechanism as well as SR Ca^{++} release mechanism in vasorelaxation produced by NTG and 3) to investigate if the coronary artery was indeed more sensitive to NTG than other peripheral arteries and veins.

Methods

Tissue preparation. Four vascular preparations were obtained from dogs: left circumflex coronary artery, superior mesenteric artery, superior mesenteric vein and saphenous vein. Male mongrel dogs, weighing 15 to 22 kg, were anesthetized with sodium brevital (approximately 150–200 mg/kg i.v.). Superior mesenteric artery, mesenteric vein and saphenous vein were carefully and rapidly excised and placed in ice-cold PSS. The heart was quickly excised and placed in chilled buffer, and the left circumflex coronary artery was isolated. All blood vessels were cleaned of fat and connective tissue and cut into 2- to 3-mm-wide rings which were equilibrated in warm (37°C) PSS and gassed with 100% O_2 for 60 to 90 min before suspending them on wire hooks. Isometric tension was recorded on a Grass model 7D polygraph connected to a computerized data acquisition system. The resting tension was: coronary and saphenous vein, 2 g; mesenteric artery and mesenteric vein, 1 g. After an initial equilibration period of 90 to 120 min, viability of each tissue was tested with 80 mM K^+-PSS (80 K^+), and tissues producing a stable contraction with a tension of at least 3 g were selected for further study. Tissues were washed and allowed to equilibrate at resting tension for 30 to 40 min before beginning all the experiments described below.

PNU-46619 contractions and NTG relaxations. Initial experiments were aimed at establishing the sensitivities of the above-mentioned four vascular preparations to PNU-46619-induced contractions and NTG-induced relaxations. First, cumulative contractions were generated in each preparation with PNU-46619 (a thromboxane analog; previously known as U-46619; 1–300 nM), with a 5-min exposure to each concentration of the agonist. Based on the CRC generated, the maximally effective concentration of PNU-46619 was selected for each vascular preparation and was as follows: saphenous vein, 30 nM; mesenteric vein, 100 nM; coronary artery and mesenteric artery, 200 nM. In a second series of experiments, maximal contraction was produced with the indicated PNU-46619 concentration, and at the plateau of the contraction (usually 15 min), cumulative relaxation responses to NTG were studied, with a 2-min exposure to each NTG concentration. NTG concentrations ranged from 1 to 3000 nM depending on the vascular preparation. All subsequent experiments were carried out with the coronary artery.

Further characterization of NTG relaxations in the coronary artery. To further establish the sensitivity of the coronary artery to NTG, cumulative relaxation CRCs to NTG were generated at three different levels of contractile activation by PNU-46619: 20 nM (~EC_{50}), 200 nM (EC_{100}) and 500 nM (supramaximal). Subsequent experiments used 200 nM PNU-46619. To study the involvement of cyclic GMP in the actions of NTG, the effect of MeB, a soluble guanylate cyclase inhibitor (Ignarro and Kadowitz, 1985), was studied. Tissues were exposed to 10 μM MeB (45 min), after which tissues were washed repeatedly with PSS to remove any free MeB left in the tissue bath. Tissues were then contracted with 200 nM PNU-46619, and NTG cumulative relaxations were studied. A control coronary ring from the same dog was used without MeB treatment. In another series of experiments, contractions were produced with a single concentration of 20, 25, 30, 50 or 80 K^+ PSS. K^+-rich PSS solutions were prepared by replacing NaCl with an equivalent amount of KCl to maintain physiological osmolality. At the plateau of the sustained high K^+ contraction, NTG (1 nM to 1 μM) cumulative relaxations were studied.

Studies with K^+ channel blockers. Experiments were carried out with ChTX (100 nM) or IbTX (200 nM), two potent and selective BK channel blockers, as well as PNU-37883A (10 μM) or PNU-99963 (100 nM), two selective K_{ATP} channel blockers (Meisheri *et al.*, 1993; Khan *et al.*, 1997). Selected experiments were also carried out with 500 nM apamin, a blocker of small conductance Ca^{++}-activated K^+ channels. Tissues were pretreated with the K^+ channel blockers 1 hr before contractions by 200 nM PNU-46619, and then cumulative relaxations to NTG were studied. For comparative purposes, cumulative relaxations were also determined with P1075 (a K_{ATP} opener), NO and ACh in selected experiments. For the study of ACh relaxation, the protocol was modified such that each tissue was tested first for the presence of endothelium; tissues that produced less than 80% relaxation of PNU-46619 contractions with 100 nM ACh were not included. Tissues then were washed, returned to resting tension and pretreated with the blocker before being recontracted with 200 nM PNU-46619 to study ACh relaxations. At least one coronary ring from each dog served as an appropriate control for each vasodilator.

Effect of NTG on PNU-46619-induced intracellular Ca^{++} release. Agonist-stimulated intracellular Ca^{++} ($[Ca^{++}]_i$) release from the SR was studied functionally as the phasic contraction induced by PNU-46619 in Ca^{++}-free PSS (EGTA-PSS) (Meisheri *et al.*, 1986, 1991). Tissues were exposed to EGTA-PSS for 15 min and contracted with PNU-46619 (200 nM), which resulted in a transient phasic contraction. When $CaCl_2$ (1.7 mM) was reintroduced in the continuing presence of PNU-46619, it resulted in a sustained contraction. In experimental tissues, NTG (1–300 nM) was added 2 min before the PNU-46619 contraction in EGTA-PSS. The peak of the PNU-46619-induced phasic contraction in EGTA-PSS was calculated as a percent of 80 K^+ contraction. Experiments were also carried out to study the influence of ChTX (100 nM, 45-min pretreatment) on the ability of NTG (30 nM) to inhibit agonist-stimulated SR $[Ca^{++}]_i$ release. For comparison, experiments also were conducted to study SR $[Ca^{++}]_i$ release inhibition by P1075 at 50 nM, its maximally effective concentration for relaxation.

Studies with RY and TG. Further characterization of the role of SR $[Ca^{++}]_i$ release in NTG vasorelaxation was studied with RY and TG, two modulators of SR Ca^{++} stores (Thastrup *et al.*, 1990; Low *et al.*, 1991; Wagner-Mann *et al.*, 1992). To select the optimal concentrations of RY and TG, initial experiments were conducted to study the concentration dependence of RY and TG (60-min pretreatment) for inhibition of 200 nM PNU-46619-stimulated SR $[Ca^{++}]_i$ release with use of the EGTA-PSS protocol described above. RY was studied at 1, 10 and 30 μM, whereas TG was studied at 0.001, 0.01, 0.1 and 1 μM. Based on these initial experiments, selected RY and TG concentrations were used to study their influence on NTG relaxations. For these experiments, tissues were pretreated with RY or TG in normal PSS for 1 hr at resting tension, contracted with 200 nM PNU-46619 in normal PSS, and cumulative NTG relaxations were studied.

Solutions and drugs. PSS contained (in mM): NaCl, 140; KCl, 4.6; $CaCl_2$ 1.5; $MgCl_2$ 1.0; glucose, 10.0; and HEPES, 5.0. The pH was adjusted to 7.3 with 1.0 N NaOH. EGTA-PSS was Ca^{++}-free PSS containing 0.2 mM EGTA, with the $MgCl_2$ concentration increased

from 1 to 1.2 mM. Drug sources were: NTG (as Tridil; DuPont, Manati, Puerto Rico); ACh (Sigma, St. Louis, MO); RY, TG and MeB (Research Biochemicals, Natick, MA); ChTX, IbTX and apamin (Peptides International, Louisville, KY); D600, P1075, PNU-37883A, PNU-46619, PNU-99963 (Pharmacia & Upjohn). A saturated solution of nitric oxide was prepared as described previously (Khan *et al.*, 1993).

Data analysis and statistics. Details of the computerized data acquisition system and customized spreadsheets used for analysis have been described previously (Khan *et al.*, 1995; Higdon *et al.*, 1997). All data are expressed as mean ± S.E.M. (n). Means and standard errors were calculated with use of the computer program EXCEL. EC_{50} values, defined as the concentration of the vasodilator that produced 50% of maximum relaxation, were calculated by NLIN2, a SAS-based program. CRCs were generated by SlideWrite *Plus*™ version 3.0. Statistical significance was determined by the Student's t test at $P = .05$.

Results

PNU-46619 contractions and NTG relaxations in different vascular preparations. Figure 1A presents cumulative CRCs for contractions induced by PNU-46619 in four dog vessels: coronary artery, mesenteric artery, mesenteric vein and saphenous vein. As shown in the figure, the mesenteric vein was the most sensitive to PNU-46619, whereas the mesenteric artery was the least sensitive of the four vessels studied. Respective EC_{50} and EC_{100} values for PNU-46619 in each of the preparations were as follows: mesenteric vein, 4.2 and 100 nM; saphenous vein, 8.2 and 100 nM; coronary artery, 25.9 and 200 nM; mesenteric artery, 76.2 and 200 nM. The magnitudes of maximal PNU-46619 contractions calculated as the percent of the first 80 mM KCl contraction in the respective blood vessels were as follows: mesenteric vein, 141%; saphenous vein, 194%; coronary artery, 85%; and mesenteric artery, 33%. Based on these data, appropriate EC_{100} PNU-46619 concentrations were chosen for the study of NTG relaxations in a given preparation. Figure 1B presents cumulative CRCs for NTG relaxations in these four preparations. Coronary artery was the most sensitive vessel, with a NTG EC_{50} of 9.4 nM. Mesenteric artery and mesenteric vein were 7- to 8-fold less sensitive to NTG with respective NTG EC_{50} values of 68.2 and 74.8 nM. Both mesenteric artery and vein also required a 10- to 30-fold higher concentration of NTG to produce maximal relaxations compared with the coronary artery (1 μM *versus* 30–100 nM in the coronary artery). Saphenous vein was least sensitive to NTG relaxations, and even 3 μM NTG produced less than 40% relaxation.

Further study of NTG relaxations in the coronary artery. Figure 2A shows the results of a study in which NTG relaxations in the coronary artery were compared at three different activation levels of PNU-46619: 20 nM (~EC_{50}), 200 nM (EC_{100}) and 500 nM (supramaximal). NTG produced

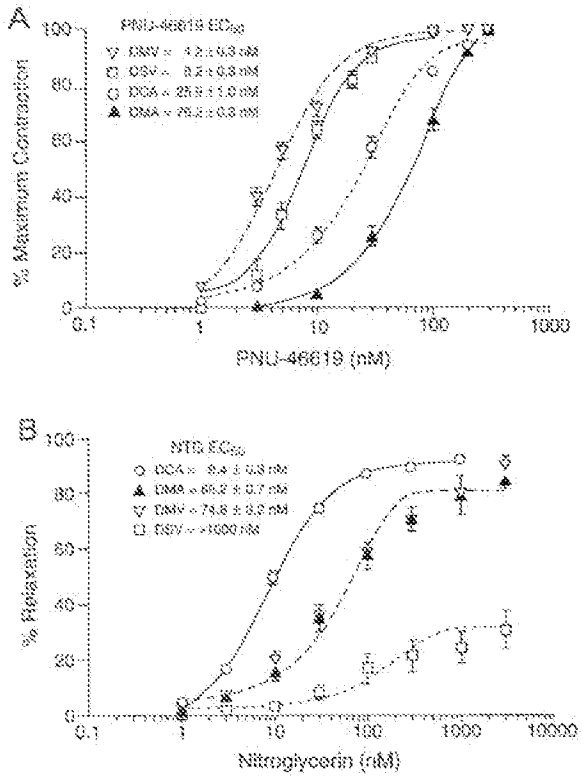

Fig. 1. (A) Cumulative CRCs for PNU-46619 in four dog vessels: mesenteric vein (DMV), saphenous vein (DSV), coronary artery (DCA) and mesenteric artery (DMA). Responses are expressed as a percent of the maximum PNU-46619 contractions (average, 3.9 g). Only one concentration-response curve was determined for each individual ring segment. Values are presented as mean ± S.E.M. from 6 to 10 ring segments from two to five dogs. (B) Cumulative relaxation CRCs for NTG in DCA, DMA, DMV and DSV precontracted with PNU-46619. Each CRC was generated using five to six ring segments from two to three dogs with the exception of NTG curve in DCA, which shows the mean ± S.E.M. from 23 rings from 14 dogs.

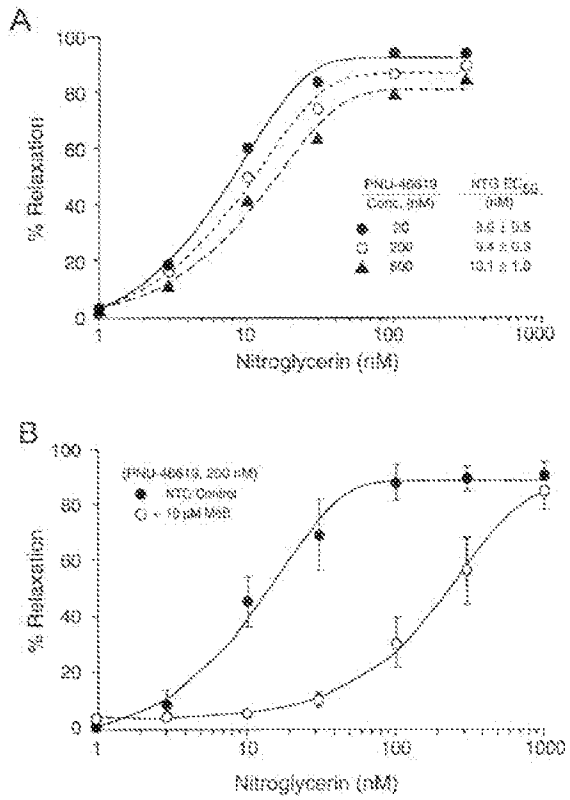

Fig. 2. (A) Cumulative relaxation CRCs for NTG in DCA precontracted with 20, 200 and 500 nM PNU-46619. Each curve was generated from five to nine coronary rings from at least four to seven dogs. Standard errors were within 10% of the mean, and the S.E.M. bars are not shown for the sake of clarity. (B) Effect of MeB on NTG relaxation CRC. After a 45-min pretreatment, MeB was washed out of the tissue for 10 min before PNU-46619 (200 nM) contraction. Each point in the CRCs represent mean ± S.E.M. of four to five rings from at least three dogs.

relaxations in the same concentration range regardless of the PNU-46619 activation level, although NTG sensitivity did diminish at higher PNU-46619 concentrations. Most NTG relaxation occurred within the 3 to 30 nM concentration range in all three cases. NTG EC_{50} values were as follows: 8.0 ± 0.5 nM (at 20 nM PNU-46619); 9.4 ± 0.3 nM (at 200 nM PNU-46619); and 10.1 ± 1.0 nM (at 500 nM PNU-46619). Subsequent studies used PNU-46619 at 200 nM to study NTG relaxations. The inhibitory effect of MeB on the NTG relaxation CRC in coronary artery is shown in figure 2B. After MeB pretreatment, no NTG relaxation could be observed up to 30 nM NTG, a concentration that is close to the EC_{50} for NTG relaxation under control conditions. Subsequent increases in NTG to 1 μM did restore maximal relaxations. Figure 3 presents results from an experiment comparing the sensitivity of NTG relaxations to different extracellular K^+, which ranged from 20 to 80 mM. NTG CRCs were shifted progressively to the right for all high K^+ contractions in comparison with the NTG CRC against agonist-induced contraction. Even at 20 mM K^+, NTG relaxations in the key concentration range of 3 to 30 nM were inhibited significantly. At 25 and 30 mM KCl, NTG relaxations up to 10 and 30 nM, respectively, were abolished. At KCl of 30 mM and above, the NTG maximal relaxation was only about 55%, even when the NTG concentration was increased up to 3 μM.

Effects of K^+ channel blockers. Figure 4A shows the effects of ChTX (100 nM) and IbTX (200 nM), two potent BK blockers, on relaxations induced by NTG (under the condition of 200 nM PNU-46619 contractions). Both ChTX and IbTX caused inhibition through the entire range of NTG CRC with NTG EC_{50} values significantly increasing from a control value of 9.4 nM to 23.3 ± 1.4 nM and 22.8 ± 1.8 nM, respectively. Increasing the concentration of ChTX to 200 nM had no further inhibitory effect on NTG relaxation (data not shown). ChTX (100 nM) had no effect on resting tension or PNU-46619 contraction, whereas IbTX (200 nM) caused approximately 20 to 30% increase in resting tension but did not increase the size of the PNU-46619 contraction. In contrast to the BK channel blockers, two K_{ATP} blockers did not have any significant inhibitory effect on NTG relaxations, as shown in figure 4B. Neither PNU-37883A (10 μM) nor PNU-99963 (100 nM) produced any significant shifts in the NTG relaxation CRCs. Data which demonstrate selectivity of the blockers are presented in figure 4C. Neither ChTX (100 nM) nor IbTX (200 nM) had any significant inhibitory effect on the relaxation CRC of P1075, a known K_{ATP} opener vasodilator. In contrast, PNU-99963 at 100 nM was a very effective blocker of P1075 relaxation (fig. 4C). The selectivity of PNU-37883A as a vascular K_{ATP} blocker was described previously (Meisheri *et al.*, 1993).

Because NTG is thought to produce its effects *via* the generation of NO, a comparative study was carried out to investigate relaxations by NO itself as well as by ACh, an

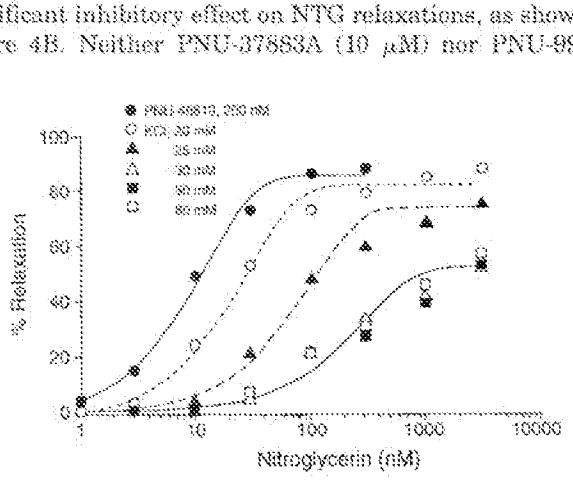

Fig. 3. NTG relaxation curves under the contraction condition of 200 nM PNU-46619 *vs.* 20, 25, 30, 50 and 80 mM KCl. Each CRC was generated from two to three rings from at least three dogs. When the coronary artery was contracted with high extracellular K^+, relaxation by NTG was diminished progressively and CRCs were shifted significantly ($P < .05$) to the right. Standard errors were within 10% of the mean and the S.E.M bars are not shown for the sake of clarity.

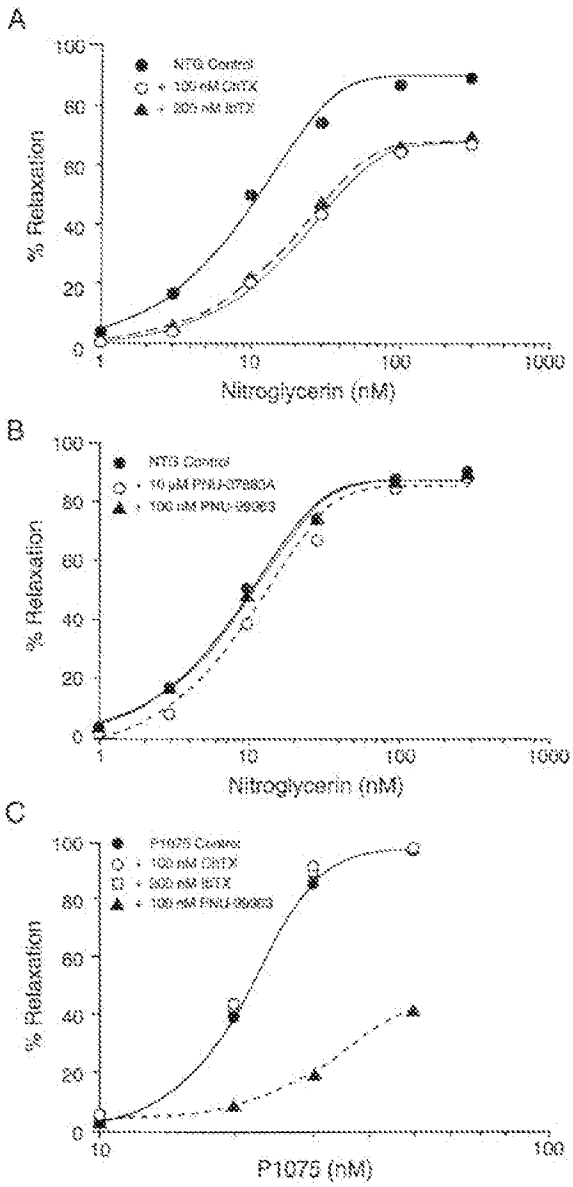

Fig. 4. (A) Effects of ChTX (100 nM) and IbTX (200 nM) on cumulative relaxation CRCs for NTG in DCA precontracted with PNU-46619 (200 nM). Each CRC was generated from at least four coronary rings from two dogs. Both ChTX and IbTX reduced the relaxation by 30 nM NTG by about 40%. (B) Effects of PNU-37883A (10 μM) and PNU-99963 (100 nM) on relaxation by NTG. Each curve was generated from five to seven rings from four to five dogs. (C) Effects of ChTX, IbTX and PNU-99963 on cumulative relaxation CRC for P1075 (10–50 nM) in DCA precontracted with PNU-46619 (200 nM). Each curve was generated from at least four rings from two dogs. In all figures, standard errors were within 10% of the mean and the S.E.M. bars are not shown for the sake of clarity.

endothelium-dependent vasodilator that releases NO from the endothelium. Figure 5 shows that coronary artery is also quite sensitive to both ACh and NO. As shown in figure 5A, the relaxation EC_{50} for ACh was 30.5 ± 2.3 nM. Pretreatment with 100 nM ChTX caused a 40% decrease ($P \leq .05$) in ACh relaxations within the range of 30 to 300 nM. As shown in figure 5B, the relaxation EC_{50} for NO was 2.7 ± 0.2 nM. Pretreatment with 200 nM IbTX caused significant ($P \leq .05$) inhibition of NO relaxations in the concentration range of 3 to 30 nM. At 3 nM NO, inhibition by IbTX was 65%, whereas at 10 nM NO, inhibition by IbTX was about 40%. In summary, NTG, ACh and NO relaxations could be distinguished clearly from that of P1075, a well established K_{ATP} opener, by their sensitivity to known BK channel blockers and their insensitivity to the known K_{ATP} blockers. In an additional experiment, apamin (500 nM), a blocker of small conductance Ca^{++}-activated K^+ channels (SK channels), had no effect on NTG relaxations in the coronary artery (data not shown). NTG CRCs from control and pretreated rings were superimposable, with identical NTG EC_{50} values of 10.1 ± 1.0 nM.

Effects of NTG on agonist-stimulated intracellular Ca^{++} release. The control (top) tracing in figure 6 shows that the phasic contraction produced by PNU-46619 in EGTA-PSS peaked in about 2 min and then declined. When extracellular $CaCl_2$ was restored in the presence of PNU-46619, the tonic contraction ensued. Phasic and tonic contractions were 1.5 ± 0.1 g and 4.4 ± .3 g, which represents 30.5 ± 1.5% and 87.0 ± 5.4%, respectively, of 80 K^+ contractions. Phasic contractions as a percent of tonic contractions were 36.2 ± 2.8%. A 2-min pretreatment with NTG, in the concentration range of 1 to 300 nM, produced a concentration-dependent inhibition of both PNU-46619-induced phasic and tonic contractions. For this study, only phasic contraction data were used, as an indication of $[Ca^{++}]_i$ release inhibition. The complete CRC for NTG inhibition of $[Ca^{++}]_i$ release is also shown in figure 6. Data are expressed as percent maximum inhibition of $[Ca^{++}]_i$ release, with 100 nM NTG data as 100% inhibition. The IC_{50} value for NTG was 8.5 ± 0.6 nM. For comparison purposes, the NTG cumulative relaxation CRC is also presented, with a NTG EC_{50} of 9.4 ± 0.3 nM. As shown in this figure, both CRCs overlap, producing statistically similar EC_{50} values. In the next experiment, the effect of ChTX on NTG-induced relaxation was compared with its effect on NTG-induced inhibition of SR $[Ca^{++}]_i$ release. As shown in figure 7A, 100 nM ChTX significantly reduced (about 40%, $P \leq .05$) the relaxation produced by 30 nM NTG. In contrast, figure 7B shows that pretreatment with 100 nM ChTX had no significant influence on the ability of 30 nM NTG to produce SR $[Ca^{++}]_i$ release inhibition. In a separate experiment, shown in figure 8A, under identical experimental conditions, 50 nM P1075 was determined to be as effective as 30 to 100 nM NTG in producing relaxation of 200 nM PNU-46619-precontracted coronary artery. This same concentration of P1075 (50 nM) was completely ineffective in inhibiting SR $[Ca^{++}]_i$ release, which was maximally inhibited by 30 to 100 nM NTG (fig. 8B). Thus, the combined data in figures 7 and 8 show that K^+ channel-mediated hyperpolarization *per se* is not important for NTG inhibition of SR $[Ca^{++}]_i$ release.

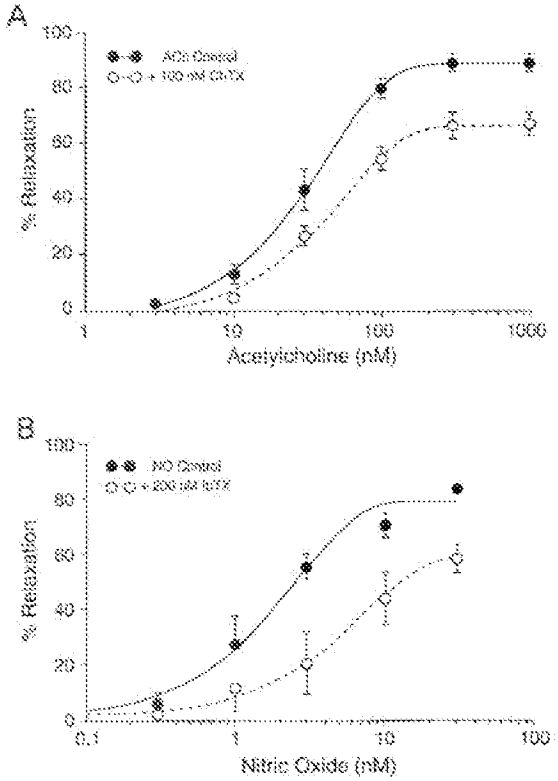

Fig. 5. Effects of 100 nM ChTX on ACh (3–1000 nM) relaxation curve (A) and 200 nM IbTX on nitric oxide (0.1–30 nM) relaxation curve (B) in DCA precontracted with PNU-46619 (200 nM). Each curve was generated from five to eight coronary rings from at least three to four dogs.

Studies with RY and TG. Figure 9A shows the concentration-dependent effect of RY on agonist-stimulated SR $[Ca^{++}]_i$ release. PNU-46619 (200 nM)-induced phasic contractions in EGTA-PSS were reduced by 66% and 82% after 1 and 10 μM RY pretreatment, respectively. No further reduction was found by increasing RY to 30 μM. After confirming that RY indeed depletes SR Ca^{++} stores, its effect on NTG relaxations was studied. Coronary rings at resting tension in normal PSS were pretreated with RY (10 μM) for 1 hr and subsequently contracted with 200 nM PNU-46619. RY significantly increased resting tension by about 50%, but had no significant effect on the magnitude of PNU-46619 contraction. Figure 9B shows that the NTG relaxation CRC was only slightly shifted to the right after RY pretreatment. RY produced a 25% and 16% reduction in NTG relaxations at concentrations of 30 and 100 nM, respectively. Associated with this, a small but significant ($P \leq .05$) increase occurred in NTG EC_{50} from a control of 13.8 to 24.9 nM.

As shown in figure 10A, TG produced significant inhibition of PNU-46619-induced SR $[Ca^{++}]_i$ release, with inhibition ranging from 42 to 71%. Figure 10B shows the effect of TG pretreatment on NTG relaxation CRCs in PNU-46619-precontracted coronary artery. Although TG pretreatment did not alter resting tension, PNU-46619 contractions were reduced significantly by 75 ± 7.6% and 63 ± 5.5% at 10 and 100 nM TG, respectively. Beginning at 10 nM, TG caused a significant shift to the right of NTG CRC and also caused significant attenuation of the maximal NTG response. Relaxation at 30 nM NTG was reduced from 66% to less than 30% after 10 nM TG treatment. This noncompetitive inhibition of NTG relaxations produced by TG was even more pronounced at 100 nM. Relaxation responses to NTG up to 30 nM essentially were eliminated, and the maximum response to NTG did not exceed 40% even after increasing NTG concentration

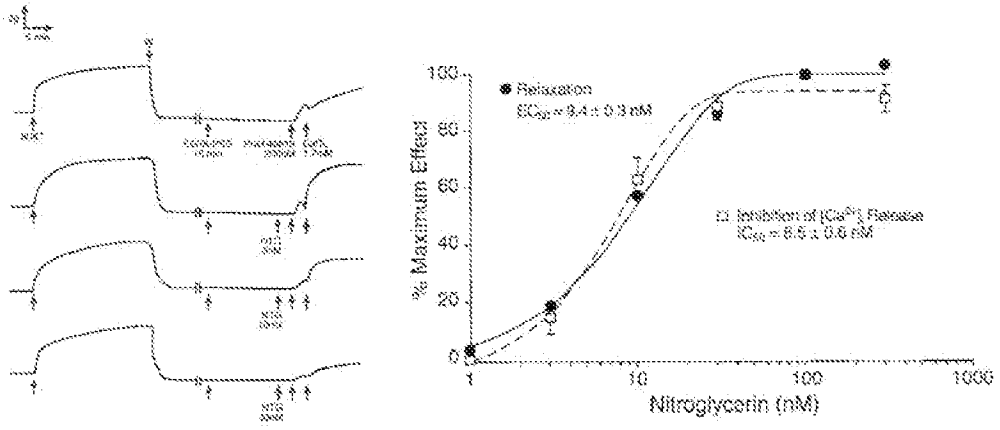

Fig. 6. Effect of NTG on PNU-46619 (200 nM)-induced $[Ca^{++}]_i$ release in DCA. Representative tracings (left). The figure on the right shows the complete CRC for inhibition of PNU-46619-induced $[Ca^{++}]_i$ release by NTG. Data are expressed as a percent maximum effect with 100 nM NTG as the maximal effective concentration. The NTG relaxation CRC was taken from previous experiments. The CRC for inhibition of $[Ca^{++}]_i$ release was obtained using 24 rings from six dogs.

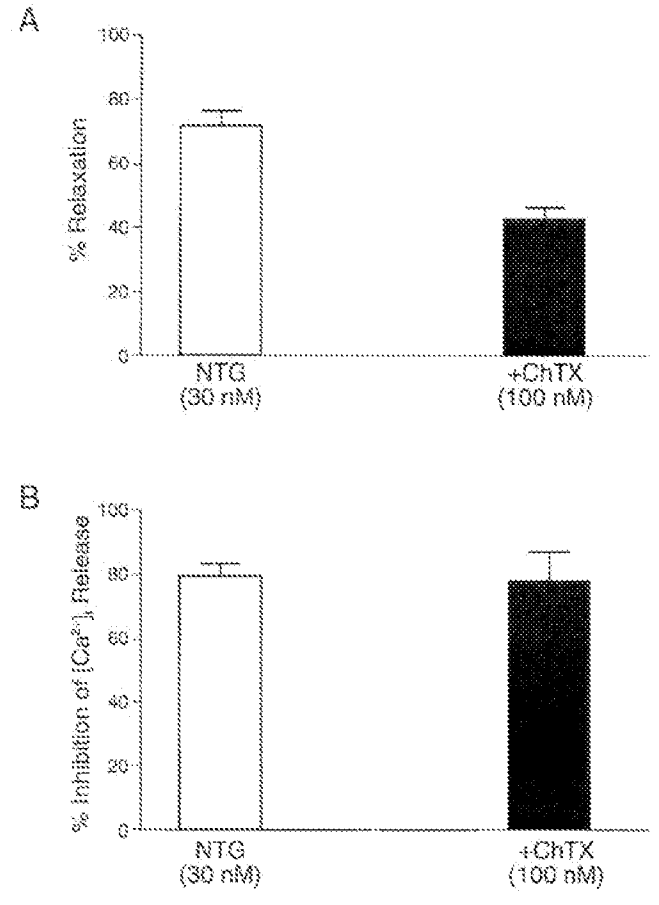

Fig. 7. (A) Effect of ChTX (100 nM) on NTG-induced relaxation of PNU-46619 (200 nM) contractions and (B) NTG inhibition of SR $[Ca^{++}]_i$ release in DCA. Each bar represents mean ± S.E.M. of 7 to 10 rings from at least seven dogs (A) and four to five rings from at least four dogs (B).

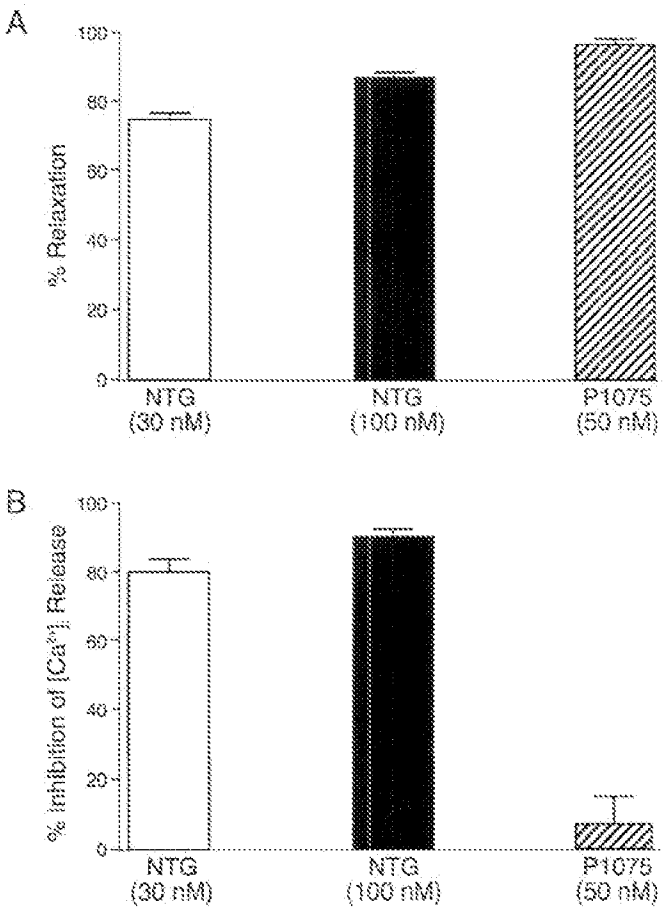

Fig. 8. Comparative effects of NTG and P1075 on PNU-46619 (200 nM)-induced contractions in DCA. (A) Bar graph showing maximum relaxation produced by NTG and P1075. Data for NTG were taken from previous studies. The bar for P1075 represents mean ± S.E.M. of four rings from two dogs. (B) P1075, at a concentration that is equipotent to NTG in producing relaxation, did not significantly inhibit SR $[Ca^{++}]_i$ release. Each bar represents mean ± S.E.M. of four to five rings from at least two dogs.

100-fold to a supramaximal level of 10 μM, TG at 1 μM caused no further inhibition of NTG CRC (data not shown).

Discussion

This study was designed to functionally evaluate the mechanisms of vasorelaxation by NTG at therapeutically relevant concentrations and in a therapeutically relevant target tissue, *i.e.*, the coronary artery. Significant findings were as follows: 1) Under similar contractile conditions, the coronary artery is significantly more sensitive to NTG than peripheral arteries or veins; 2) NTG relaxations are attenuated significantly under conditions that limit K^+ gradients across the plasma membrane and also by the use of selective BK channel blockers; 3) NTG is a potent inhibitor of agonist-stimulated SR $[Ca^{++}]_i$ release, and this effect is independent of membrane BK channel activation and hyperpolarization *per se*; and 4) NTG relaxation is not altered by blockade of the SR Ca^{++} release channel by RY, but significantly attenuated by the blockade of the SR Ca^{++}-ATPase pump by TG.

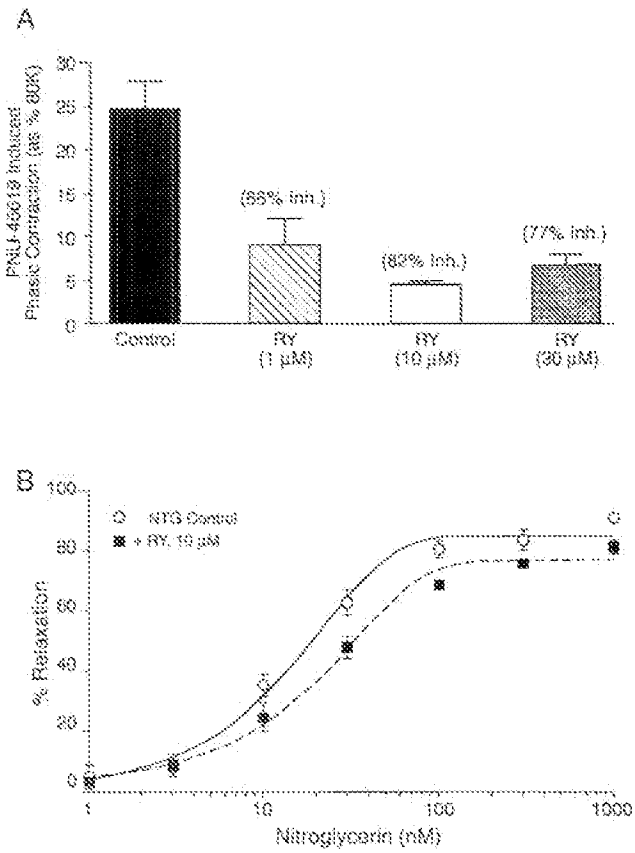

Fig. 9. (A) Effect of RY on 200 nM PNU-46619-induced phasic contractions in DCA. RY produced significant ($P \leq .05$) inhibition (Inh.) of PNU-46619-induced phasic contractions at 1, 10 and 30 μM. Each bar represents mean ± S.E.M. of three to six rings from at least three dogs. (B) Effect of RY on NTG relaxation CRC in DCA precontracted with PNU-46619 (200 nM). Each point of the CRCs represents mean ± S.E.M. of four to five rings from three dogs.

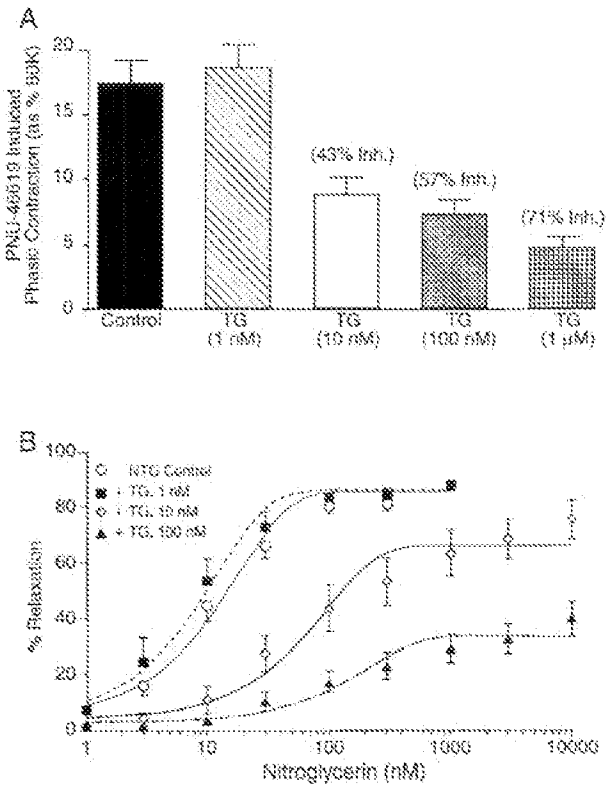

Fig. 10. (A) Effect of TG on PNU-46619 (200 nM)-induced phasic contractions in DCA. TG produced a concentration-dependent inhibition of PNU-46619-induced phasic contractions, with significant ($P \leq .05$) inhibitions at 10 nM, 100 nM and 1 μM. Each bar represents mean ± S.E.M. of three to eight rings from at least three dogs. (B) Effect of TG on NTG relaxation CRC in DCA precontracted with PNU-46619 (200 nM). Each point in the CRCs represents mean ± S.E.M. of four to seven rings from at least four dogs.

Although a large database is available for NTG relaxations in various vascular preparations, a comparative study of the sensitivity of various blood vessels to NTG under fairly controlled contractile conditions in the same study has not been reported previously. When tissues were contracted to a similar contractile level by the same agonist, the coronary artery was clearly the most sensitive vascular preparation. In general, peripheral vascular preparations demonstrate low sensitivity to NTG when tissues are maximally contracted with an agonist. Typical NTG EC_{50} values reported have been in the 100 nM range (Miwa and Toda, 1985; Mackenzie and Parratt, 1977; Khan *et al.*, 1993), whereas we found that the NTG EC_{50} in the coronary artery was approximately 10 nM. The basis for this differential sensitivity to NTG is most likely multifactorial. As will be discussed later, NTG relaxation involves effects on both plasmalemmal Ca^{++} influx as well as intracellular Ca^{++} stores. Because agonist-induced contractions use various Ca^{++} sources for contraction to different degrees in different vascular preparations, an important rationale is formed for differential sensitivity to various vasodilators including NTG (Cauvin *et al.*, 1984). Further investigations into the basis of these differences would be of interest. The remainder of the present study was aimed at delineating the mechanisms involved in coronary relaxations by NTG.

The first evidence of the importance of K^+ channel-mediated hyperpolarization in the actions of NTG was provided by the differential potency of NTG in relaxing agonist-induced contractions *versus* 20 to 80 mM KCl-PSS-induced contractions. Vasodilators dependent on the K^+ channel mechanism lose their effects when exposed to high K^+ solutions because an increase in extracellular K^+ attenuates the K^+ gradient across the plasma membrane, thus rendering the K^+ channel-activating mechanism ineffective. When the coronary artery was contracted with high extracellular K^+, relaxation by NTG was reduced progressively and CRC was shifted to the right. At 30 mM KCl, inhibition was so pronounced that even a 30-fold increase in the NTG concentration could not restore maximal relaxations. Because the high K^+ condition can produce multiple effects, a more direct pharmacological approach was taken by the use of BK channel blockers. ChTX and IbTX are highly selective blockers that inhibit high-conductance K_{Ca} in smooth muscle and neuroendocrine tissues (Garcia *et al.*, 1991). Selective inhibitory effects of these blockers on cyclic GMP vasodilators in bovine tracheal smooth muscle and rabbit mesenteric artery have been reported previously (Hamaguchi *et al.*, 1992; Khan *et al.*, 1993). Thus, in a clinically relevant concentration range of NTG (3–30 nM), a significant relaxation component appears to be highly sensitive to blockade by BK channel blockers. It was also demonstrated that NTG-induced relaxation was not attenuated by K_{ATP} channel blockers (PNU-37883A and PNU-99963). Relaxations by P1075, a K_{ATP} opener, on the other hand, were very sensitive to blockade by PNU-99963, a re-

cently discovered potent cyanoguanidine K_{ATP} blocker (Khan *et al.*, 1997). These data collectively show that NTG is distinct from K_{ATP} opener vasodilators. The lack of an effect of ChTX and IbTX on P1075 relaxations also demonstrates the pharmacological selectivity of these BK channel blockers in the coronary artery. Finally, comparative studies with NTG, NO and ACh show that BK channel blockers produce significant inhibition of relaxations by all three agents in the coronary artery. Thus, BK channel activation apparently is a key mechanism for coronary artery relaxation by cyclic GMP-mediated vasodilators such as, NTG, ACh and NO. Collectively, these data support the electrophysiological evidence for BK channel activation by the cyclic GMP system in the coronary artery (Taniguchi *et al.*, 1993). However, these studies noted that a significant portion of relaxation still existed after BK channel blockade, which suggests that an additional mechanism(s) is likely involved in NTG relaxation.

Another important mechanism involved in the action of cyclic GMP-increasing vasodilators is the SR Ca^{++} stores (Meisheri *et al.*, 1986; Lincoln and Cornwell, 1991). Vascular SR Ca^{++} stores are important as modulators of cellular Ca^{++} homeostasis and for regulation of Ca^{++} concentrations for smooth muscle contractions (Sturek *et al.*, 1992; Van Breemen *et al.*, 1995; Golovina and Blaustein, 1997). The present study shows that NTG concentration-dependently inhibited PNU-46619-induced SR $[Ca^{++}]_i$ release with an IC_{50} value of about 10 nM, which is identical to the EC_{50} for NTG relaxation. We have shown further that the effects of NTG on SR Ca^{++} stores are independent of BK channel activation, because ChTX did not attenuate SR $[Ca^{++}]_i$ release inhibition by NTG. These data, combined with the observation that P1075 does not cause inhibition of SR $[Ca^{++}]_i$ release, suggest the lack of a causal relationship between hyperpolarization and inhibition of agonist stimulated SR $[Ca^{++}]_i$ release.

Further definition of the mechanism used by NTG to produce its effects on SR Ca^{++} stores came from the use of RY and TG. RY causes irreversible opening of SR Ca^{++} release channels thereby causing a depletion of the SR (Low *et al.*, 1991; Wagner-mann *et al.*, 1992), whereas TG, a potent inhibitor of the SR Ca^{++}-ATPase pump, prevents the ability of SR to take up Ca^{++} and thus depletes SR (Thastrup *et al.*, 1990). Although both agents caused a similar degree of SR Ca^{++} store depletion, their effects on NTG-induced relaxation were quite distinct. In the presence of RY, NTG still retained most of its ability to cause relaxation of the coronary artery, which suggests that the SR Ca^{++} release channel is not the primary site of action of NTG. In contrast, TG caused a pronounced loss of relaxation by NTG, pointing toward a role of the SR Ca^{++}-ATPase pump. This apparently is the first study providing such a clear-cut demonstration of the differential modulation of NTG relaxation by agents that modify SR Ca^{++} store function. The high sensitivity of NTG to TG strongly suggests that the SR Ca^{++}-ATPase pump is the primary pharmacological target for the actions of NTG, and this most likely is mediated *via* the cyclic GMP pathway. In support of this observation, a biochemical database is available which demonstrates that the smooth muscle SR Ca^{++}-ATPase is a key target for phosphorylation by cyclic GMP-dependent protein kinase (Cornwell *et al.*, 1991; Lincoln and Cornwell, 1993).

A schematic diagram providing the sequence of events that are likely involved in the actions of NTG on the coronary artery is presented in figure 11. Pharmacological evidence has been presented in this study to support most of the key steps outlined in this diagram. Overall, this study provides evidence to support the concept that nitrovasodilators produce clinically relevant coronary vasorelaxation by primarily affecting two cellular mechanisms *via* a cyclic GMP pathway: 1) activation of plasmalemmal BK channels which would lead to hyperpolarization-induced inhibition of Ca^{++} entry *via* the voltage-gated Ca^{++} channels, and 2) activation of SR Ca^{++}-ATPase pump, which would lead to enhanced accumulation of Ca^{++} in the intracellular stores. Together, both of these actions would lead to decreased cytoplasmic free Ca^{++} concentration to produce relaxation. Both of these mechanisms appear to be equally important in the actions of NTG, and this characteristic may be responsible for the unique vasorelaxation profile produced by NTG-type vasodilators.

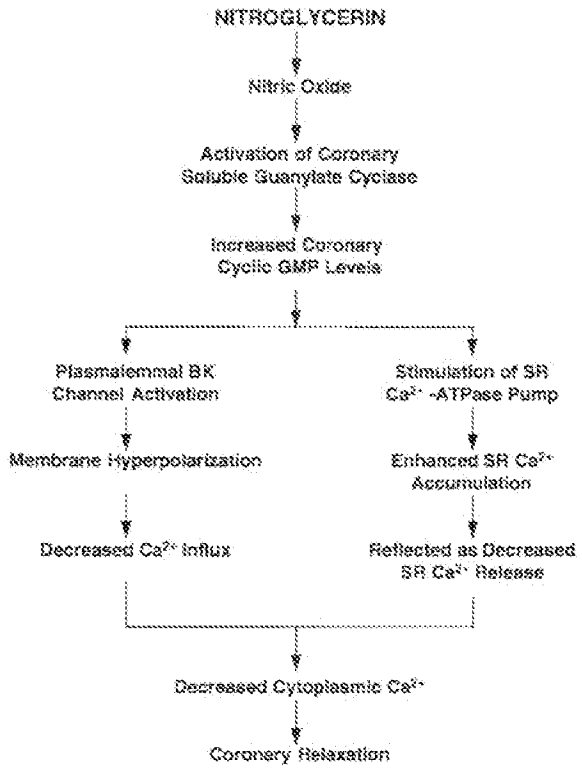

Fig. 11. Schematic diagram illustrating the possible mechanisms of action of NTG as a coronary vasodilator.

Acknowledgments

We greatly appreciate the assistance of Lew V. Buchanan of Pharmacia and Upjohn (PNU) for sacrificing dogs for the retrieval of tissues. We would like to thank Dr. W. R. Mathews of PNU for assisting and allowing us to use the facility of his laboratory for the preparation of nitric oxide solution.

References

Cauvin C, Lukeman S, Cameron J, Hwang O, Meisheri K, Yamamoto R and Van-Breeman C (1984) Theoretical bases for vascular selectivity of Ca^{++} antagonists. *J Cardiovasc Pharmacol* **6**(6):S630–S638.

Cornwell TL, Pryzwansky KB, Wyatt TA and Lincoln TM (1991) Regulation of sarcoplasmic reticulum protein phosphorylation by localized cyclic GMP-dependent protein kinase in vascular smooth muscle cells. *Mol Pharmacol* **40**:923–931.

Garcia ML, Galvez A, Garcia-Calvo M, King VF, Vazquez J and Kaczorowski GJ (1991) Use of toxins to study potassium channels. *J Bioenerg Biomembr* **23**:615–646.

Golovina VA and Blaustein MP (1997) Spatially and functionally distinct Ca^{2+} stores in sarcoplasmic and endoplasmic reticulum. *Science* **275**:1643–1648.

Hamaguchi M, Ishibashi T and Imai S (1992) Involvement of charybdotoxin-

sensitive K^+ channel in the relaxation of bovine tracheal smooth muscle by glyceryl trinitrate and sodium nitroprusside. *J Pharmacol Exp Ther* **262**:265–270.
He G, Yang C, Gately H, Kernaey A, Swanson J, Ahmad A, Fisten S, Wood J and Starr A (1996) Potential greater than additive vasorelaxant actions of milrinone and nitroglycerin on human conduit arteries. *Br J Clin Pharmacol* **41**:101–107.
Hester RK (1985) Effects of 2-nicotinamidoethyl nitrate on agonist sensitive Ca^{++} release and Ca^{++} entry in rabbit aorta. *J Pharmacol Exp Ther* **233**:108–111.
Higdon NR, Khan SA, Ruchusan LV and Meisheri KD (1997) Tissue and species variation in the vascular receptor binding of ^{3}H-P1075, a potent K_{ATP} opener vasodilator. *J Pharmacol Exp Ther* **280**:255–260.
Hirata M, Kohse KP, Chang C, Ikebe T and Murad F (1990) Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. *J Biol Chem* **265**:1268–1273.
Ignarro LJ and Kadowitz PJ (1985) The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annu Rev Pharmacol Toxicol* **25**:171–191.
Kaeaki H, Murakami K and Urakawa N (1986) Mechanism of inhibitory action of sodium nitroprusside in vascular smooth muscle of rabbit aorta. *Arch Int Pharmacodyn* **280**:230–240.
Khan SA, Mathews WR and Meisheri KD (1993) Role of calcium-activated K^+ channels in vasodilation induced by nitroglycerine, acetylcholine and nitric oxide. *J Pharmacol Exp Ther* **267**:1327–1335.
Khan SA, Higdon NR, Hester JB and Meisheri KD (1997) Pharmacological characterization of novel cyanoguanidines as vascular K_{ATP} blockers. *J Pharmacol Exp Ther* **283**:1207–1213.
Lincoln TM (1989) Cyclic GMP and mechanisms of vasodilation. *Pharmacol Ther* **41**:479–502.
Lincoln TM and Cornwell TL (1991) Towards an understanding of the mechanism of action of cyclic AMP and cyclic GMP in smooth muscle relaxation. *Blood Vessels* **28**:129–137.
Lincoln TM and Cornwell TL (1993) Intracellular cyclic GMP receptor proteins. *FASEB J* **7**:328–338.
Low AM, Gasper V, Kwan CY, Darby PJ, Bourreau JP and Daniel EE (1991) Thapsigargin inhibits repletion of phenylephrine-sensitive intracellular Ca^{++} pool in vascular smooth muscle. *J Pharmacol Exp Ther* **258**:1105–1113.
Mackenzie JE and Parratt JR (1977) Comparative effects of glyceryl trinitrate on venous and arterial smooth muscle *in vitro*; relevance to antianginal activity. *Br J Pharmacol* **60**:155–160.
Meisheri KD, Taylor CJ and Sasaki H (1986) Synthetic atrial peptide inhibits intracellular Ca^{++} release in smooth muscle. *Am J Physiol* **250**:C171–C174.
Meisheri KD, Cipkus-Dubray LA, Rosner JM and Khan SA (1991) Nicorandil-induced vasorelaxation: functional evidence for K^+ channel-dependent and cyclic GMP-dependent components in a single vascular preparation. *J Cardiovasc Pharmacol* **17**:909–912.
Meisheri KD, Khan SA and Martin JL (1993) Vascular pharmacology of ATP-sensitive K^+ channels: Interaction between glyburide and K^+ channel openers. *J Vasc Res* **30**:2–12.
Miwa K and Toda N (1985) The regional differences of relaxations induced by various vasodilators in isolated dog coronary and mesenteric arteries. *Jpn J Pharmacol* **38**:318–320.
Sturek M, Kunda K and Hu Q (1992) Sarcoplasmic reticulum buffering of myoplasmic calcium in bovine coronary smooth muscle. *J Physiol* **453**:25–48.
Taniguchi J, Furukawa KI and Shigekawa M (1993) $Maxi\text{-}K^+$ channels are stimulated by cyclic guanosine monophosphate-dependent protein kinase in canine coronary artery smooth muscle cells. *Pflugers Arch* **423**:167–172.
Tare M, Parkington HC, Coleman HA, Neild TO and Dusting GJ (1990) Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature (Lond)* **346**:69–71.
Taylor CJ and Meisheri KD (1986) Inhibitory effects of a synthetic atrial peptide on contractions and ^{45}Ca fluxes in vascular smooth muscle. *J Pharmacol Exp Ther* **237**:803–808.
Thastrup O, Cullen PJ, Drobak BK, Hanley MR and Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} ATPase. *Proc Natl Acad Sci USA* **87**:2466–2470.
Van Breemen C, Chen Q and Laher I (1995) Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. *Trends Pharmacol Sci* **16**:98–105.
Wagner-Mann C, Hu Q and Sturek M (1992) Multiple effects of cyanidine on intracellular free Ca^{2+} in smooth muscle cells from bovine and porcine coronary artery: modulation of sarcoplasmic reticulum function. *Br J Pharmacol* **105**:903–911.
Wei JY and Saed FR (1979) Quantitative determination of trinitroglycerin in human plasma. *Circulation* **59**:588–592.
Yoshida Y, Sun H, Cai J and Imai S (1991) Cyclic GMP-dependent protein kinase stimulates the plasma membrane Ca^{2+} pump ATPase of vascular smooth muscle via phosphorylation of a 240-kDa protein. *J Biol Chem* **266**:19819–19827.

Send reprint requests to: Sajida A. Khan, Pharmacology, 7250–209-315, Pharmacia & Upjohn Inc., Kalamazoo, MI 49001. e-mail: sakhan@am.pnu.com